W. P., Proc. Soc. Exptl. Biol. Med. 112, 601 (1963).
3. Hsiung, G. O., Fischman, H. R., Fung, C. K. Y., and Green, R. H., Proc. Soc. Exptl. Biol. Med. 130, 80 (1969).
4. Kuttner, A. G. and Wang, S. H., J. Exptl. Med. 60, 773 (1934).
5. Plummer, G. and Benyesh-Melnick, M., Proc. Soc. Exptl. Biol. Med. 117, 145 (1964).

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Nucleic Acid Content of Visna Virus* (34011)

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Visna virus produces a progressive and fatal neurological disease of sheep (1, 2). Many months or years may pass between introduction of the virus into the animal and the first appearance of disease, which establishes the "slow" nature of the infection.

Visna virus was first propagated in primary cell structures prepared from sheep choroid plexus (SCP) (3), and most studies of the virus have been performed with such cultures. In SCP cells virus growth occurs after a latent period of 16–20 hr; infective virus increases exponentially from 20–36 hr, followed by a gradual increment until 72–96 hr after infection (4, 5). Virus replication is accompanied by cytopathic changes and cell destruction.

Visna virus has also been shown to multiply in a continuous cell line derived from pig kidney [PK(15)] (6). Peak titers of infective virus in these cells are reached 96–168 hr after infection, and neither cytopathic changes nor cell depletion are observed.

Electron microscopic observations indicate that virus particles are formed by budding from the cytoplasmic membrane of host cells in a manner similar to the RNA-containing avian leukosis and murine leukemia viruses (7), and histochemical observations demonstrate that virus-infected cells harbor increased amounts of cytoplasmic RNA (8) and contain aggregates of viral antigen which have the staining characteristics of RNA (9). Although these data strongly suggest that visna virus is an RNA-containing virus, direct evidence of the nature of the nucleic acid in the infective virus particle is still lacking.

This report describes the purification of visna virus by density-gradient centrifugation in cesium chloride, attempts to incorporate radioactive nucleic acid precursors into virus particles, and studies on the effect of actinomycin D on RNA synthesis in virus-infected cells. The results indicate that visna virus contains RNA and suggest that actinomycin D suppresses virus-specific RNA synthesis.

Materials and Methods. Cell cultures. Sheep choroid plexus (SCP) cells were prepared by trypsin dispersion of choroid plexuses removed from the brains of exsanguinated domestic Hampshire or Suffolk sheep as previously described (5). Cells were grown in reinforced Eagle's medium (10) containing 10% fetal bovine serum in 250-ml plastic flasks and incubated at 37°. SCP cells in their fourth serial pasage were suspended in medium containing glycerin, frozen and stored in a liquid nitrogen refrigerator; cells were reconstituted when needed and used in their sixth to ninth passage.

Porcine kidney [PK(15)] cells were obtained from the American Type Culture Col-

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lection Cell Repository in their 132nd passage and grown in reinforced Eagle's medium containing 10% fetal bovine serum.

Virus. Visna virus K485 was obtained from H. Thormar and P. A. Pálsson, Institute of Experimental Pathology, University of Iceland, and was carried through twelve serial passages in SCP cells. Twelfth-passage virus containing $8.4 \times 10^{5.0}$ $TCID_{50}$/ml was used in the experiments.

Infection of cell cultures. Confluent SCP monolayers grown in plastic tissue culture dishes containing $6.0–9.0 \times 10^5$ cells were washed with phosphate-buffered saline (PBS), pH 7.2 (11), and infected with virus at a multiplicity of 0.2–0.5 $TCID_{50}$/cell. Such virus/cell ratios were chosen to avoid the destructive effects of rapid cell fusion which are observed when inocula containing larger quantities of virus are used (5).

After an adsorption period of 2 hr at 37°, the virus inoculum was removed and the cell sheet was again washed with PBS. Infected cultures were maintained at 37° in reinforced Eagle's medium containing 2% heat-inactivated lamb serum in a humidified atmosphere of 5% carbon dioxide.

Control uninfected SCP monolayers were treated in a similar manner but were inoculated with reinforced Eagle's medium and 0.5% bovine plasma albumin (BSA, Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.).

Growth of radioactively labelled virus. Infected and control SCP monolayers in 100-mm plastic dishes were maintained in reinforced Eagle's medium and 2% lamb serum containing 15 μCi ^{3}H-uridine/ml, 15 μCi ^{3}H-thymidine/ml or 4 μCi ^{32}P-orthophosphate/ml. Twenty-four, 46, 54, 69, 77, 100, and 142 hr after infection, the medium was removed and fresh medium containing radioactive precursors was added. Harvested medium was stored at 4° until used for virus purification.

Purification of virus. The medium from virus-infected cell cultures was clarified at 8,700*g* for 15 min at 4° and concentrated at 78,000*g* for 2 hr. Pelleted virus was suspended in one-hundredth of its original volume in a solution containing 100 μg each of deoxyribonuclease and ribonuclease in PBS containing 0.01 *M* Mg^{2+}, held at 4° for 12 hr, and incubated at 37° for 1 hr. The virus is resistant to these enzymes (3). Virus concentrated in this manner contained $6.3 \times 10^7 – 2.0 \times 10^9$ $TCID_{50}$/ml.

Concentrated virus (0.5 ml) was then carefully layered on 4.0 ml of 1.58 *M* cesium chloride in 0.1 *M* phosphate buffer, pH 7.5, containing 0.001 *M* EDTA (density 1.20 gm/ml) in a Lusteroid tube and centrifuged at 178,000*g* in a Spinco SW 65 swinging bucket rotor for 24 hr at 4°. Ten-drop fractions were collected from below using a Beckman fractionating system. The density of selected fractions was determined by weighing 100-μl samples in calibrated micropipettes on an analytical balance.

Assay of infective virus. Infective visna virus was assayed on confluent SCP cell monolayers in 60-mm plastic dishes by the method previously described (5).

Radioactivity determinations. Gradient fractions were precipitated with trichloroacetic acid (TCA, final concentration 5%). The precipitates were collected on nitrocellulose filter discs (B-6 Schleicher & Schuell Co., Keene, N.H.) and washed extensively with 5% TCA. The discs were dried and placed in vials containing 10 ml of scintillation fluid (4 g Omnifluor per liter of toluene). Retained radioactivity was determined in a refrigerated Packard liquid scintillation spectrometer.

Incorporation experiments. Studies on the incorporation of ^{3}H-uridine into SCP or PK(15) cells were carried out by the coverslip technique (12). Confluent cell monolayers were grown in 60-mm plastic tissue culture dishes containing 4–6 15-mm circular coverslips. Each coverslip contained $2.6 \times 10^{4.0}$ SCP or $2.2 \times 10^{5.0}$ PK(15) cells.

At various times after infection, the cells were treated with actinomycin D for 2 hr and then exposed to ^{3}H-uridine at a concentration of 25–40 μCi/ml. Incorporation was stopped after 60 min by rapid fixation of the cells in cold acetic acid–alcohol (3 parts absolute ethanol to 1 part glacial acetic acid). Coverslips were then collected, extracted with

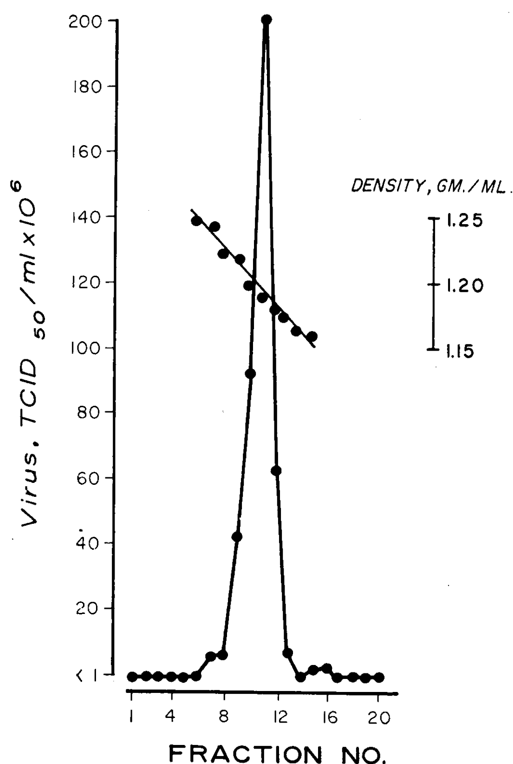

FIG. 1. Equilibrium density-gradient centrifugation of infective visna virus in cesium chloride. Virus suspension, partially purified by differential centrifugation, was placed on a cesium chloride solution of density 1.20 g/ml and centrifuged at 178,000*g* for 24 hr at 4°.

5.0% perchloric acid, washed with ether–ethanol, dried, and assayed for radioactivity in vials containing 10 ml of scintillation fluid.

Chemicals and isotopes. Analytical grade cesium chloride was obtained from Stanley Cohen & Co., Yonkers, N.Y.; five-times crystallized ribonuclease and once-crystallized deoxyribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo. Actinomycin D was a gift from Merck, Sharp & Dohme, Rahway, N.J. 5-^{3}H-uridine (26.6 Ci/mmole), ^{3}H-methyl-thymidine (6.7 Ci/mmole), and Omnifluor were products of the New England Nuclear Corporation. ^{32}P-orthophosphate was purchased from E. R. Squibb and Sons, New York.

Results. Distribution of infectivity in density gradients. The distribution of infective visna virus in a cesium chloride density gradient is shown in Fig. 1. Ninety-four per cent of infective virus in the gradient was recovered in four fractions banding at a density between 1.18 and 1.20 g/ml. The fraction containing the maximal amount of infective virus (47% of total) had a density of 1.19 g/ml.

The band of infective virus corresponded to a homogenous white zone approximately 18–20 mm from the bottom of the tube. In addition, two white flaky bands were observed 2–4 mm from the bottom of the tube at a density of 1.30–1.32 g/ml; these bands did not contain significant amounts of infective virus.

These results are similar to previously reported observations on the sedimentation of visna virus in equilibrium density gradients of potassium tartrate (5).

Distribution of ^{3}H-nucleosides and ^{32}P in density gradients. Medium containing radioactive ^{3}H-uridine or ^{32}P-orthophosphate derived either from visna virus–infected cells or from control uninfected SCP cultures was concentrated and purified in CsCl density gradients.

The distribution of ^{3}H-uridine is shown in Fig. 2. Two radioactive peaks were present in gradients of concentrated virus preparations. The lower peak corresponded to the heavier flaky bands, which did not contain significant amounts of infectious virus; the upper peak corresponded to the fractions that contained infectious virus.

In gradients of medium concentrated from uninfected cultures, a single smaller radioactive peak was also found; it corresponded to the heavier bands. No radioactivity was detected in fractions sedimenting at the density at which infective visna virus particles are found.

Attempts to introduce ^{3}H-thymidine into infective virus were unsuccessful. As shown in Fig. 3, density-gradient centrifugation of virus concentrate from SCP cultures maintained in medium containing ^{3}H-thymidine failed to produce a radioactive peak in the infective virus zone.

Similar results were obtained with ^{32}P-labeled virus and control harvests (Fig. 4).

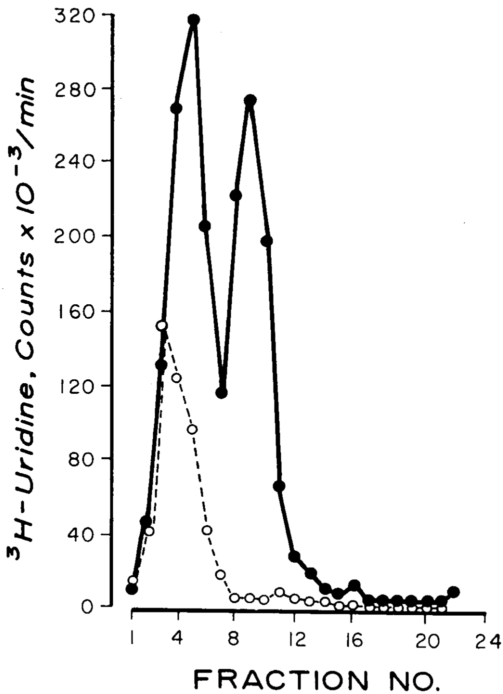

FIG. 2. Distribution of ^{3}H in equilibrium density gradients of concentrated harvests from visna-infected (●——●) and uninfected (O- - -O) SCP cell cultures maintained in medium containing ^{3}H-uridine. Cesium chloride density gradient centrifuged at 178,000g for 24 hr at 4°.

Two ^{32}P peaks were observed in cesium chloride gradient centrifugation of concentrates from infected cultures: one corresponded to the fractions containing infective virus and the other was associated with flaky material which banded in the lower portion of the tube. Cesium chloride gradients of ^{32}P-labeled harvests from uninfected cultures did not contain significant peaks of radioactivity.

The results indicate that infective visna virus particles incorporate ^{3}H-uridine and ^{32}P, but not ^{3}H-thymidine, and suggest that visna virus is an RNA-containing virus.

Effect of actinomycin D on RNA synthesis in SCP and PK (15) cells. Certain tissue culture cell lines show unusual resistance to the suppressive effect of actinomycin D on RNA synthesis (13, 14). Experiments were therefore performed to test the susceptibility of SCP and PK(15) cells to actinomycin D. As indicated in Table I, ^{3}H-uridine incorpo-

TABLE I. Effect of Actinomycin D on the Incorporation of ^{3}H-Uridine by SCP and PK(15) Cells.

Actinomycin D (μg/ml)	^{3}H-uridine (cpm/coverslip)[a]		Percentage of inhibition	
	SCP cells	PK(15) cells	SCP cells	PK(15) cells
0	50,777	66,627	—	—
0.2	1039	3199	98	95
2.0	198	442	99	99
4.0	177	288	99	99
20.0	249	227	99	99

[a] Monolayers were exposed to actinomycin D for 2 hr and then exposed to ^{3}H-uridine (10 μCi/ml) for 1 hr. Coverslips were then fixed, extracted with perchloric acid, and assayed for radioactivity.

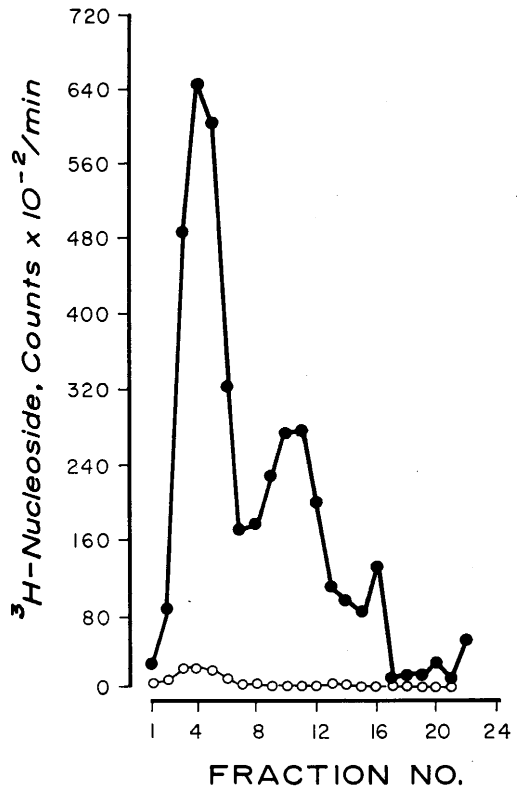

FIG. 3. Distribution of ^{3}H in equilibrium density gradients of infective visna virus grown in SCP cells in the presence of ^{3}H-uridine (●——●) or ^{3}H-thymidine (O——O). Cesium chloride density gradient centrifuged at 178,000g for 24 hr at 4°.

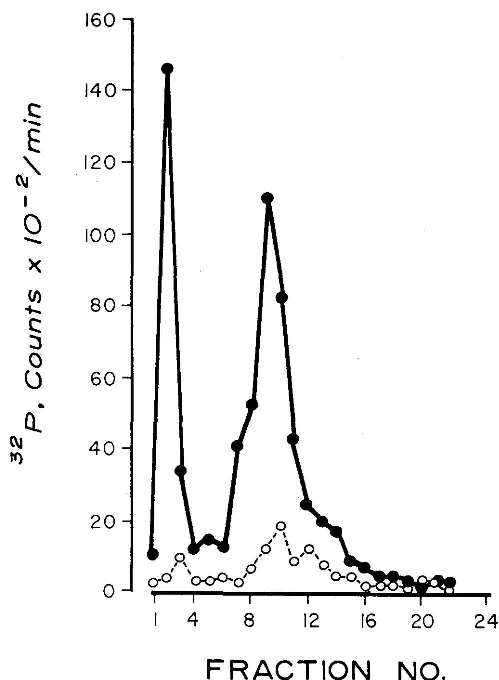

FIG. 4. Distribution of ^{32}P in equilibrium density gradients of concentrated harvests from visna-infected (●——●) and uninfected (O- - -O) SCP cell cultures maintained in medium containing ^{32}P. Cesium chloride density gradient centrifuged at 178,-000g for 24 hr at 4°.

ration into both cell lines was reduced to 1% of control values by actinomycin D in concentrations of 2.0 μg/ml or greater. No cytopathic changes were detected in SCP or PK(15) cells after 2 hr of exposure to actinomycin D at these concentrations.

The results indicate that SCP or PK(15) cells are not unusually resistant to the inhibitory effect of actinomycin D on RNA synthesis.

Effect of actinomycin D on RNA synthesis in visna virus–infected SCP and PK(15) cells. Experiments were performed to determine whether actinomycin D could be used to unmask virus-induced RNA synthesis in cells actively producing visna virus. At intervals after infection, medium was removed from infected cell cultures and assayed for infective virus. Fresh maintenance medium containing actinomycin D was added and after a 2-hr exposure to actinomycin D, ^{3}H-uridine was introduced into the culture fluid. One hour later the coverslip preparations were fixed, extracted, and assayed for radioactivity. A similar procedure was employed using control uninfected cultures.

The results of experiments with SCP cells are given in Table II. The incorporation of ^{3}H-uridine into infected cultures exceeded that of uninfected cultures only at 4 and 24 hr after infection, and the difference at 24 hr was minor. At other time points, ^{3}H-uridine incorporation in infected cells was significantly less than in the controls. Similar results were obtained using actinomycin D at a lower concentration (0.2 μg/ml.).

Cytopathic changes in SCP cells caused by visna virus were first detected 24–30 hr after infection. By 48–54 hr after infection a significant number of cells became detached from the glass surface. It was, therefore, possible that the decline in incorporation of ^{3}H-uridine in visna virus–infected SCP cultures resulted from depletion of the cell population due to virus-induced cytopathic effects.

To overcome this difficulty, similar ^{3}H-uridine incorporation experiments were performed on actinomycin D–treated infected and uninfected PK(15) cells. As previously

TABLE II. Effect of Actinomycin D on the Incorporation of ^{3}H-Uridine into Visna Virus–Infected SCP Cells.

Time after infection (hr)	Infective virus[a] ($TCID_{50}$/ml)	^{3}H-uridine (cpm coverslip)[b] In-fected	Control	Per-centage of control
4	6.3×10^{3}	277	200	139
24	1.0×10^{4}	760	696	109
48	8.8×10^{5}	293	396	74
72	2.9×10^{7}	304	654	47
96	2.0×10^{7}	160	386	42
120	2.0×10^{6}	100	160	62

[a] Released virus present in medium prior to addition of actinomycin D.

[b] At the times indicated, monolayers were incubated with 2.5–4.0 μg actinomycin D per ml for 2 hr at 37° followed by the addition of ^{3}H-uridine at a concentration of 25–45 μCi/ml for 1 hr. Coverslips were then fixed, extracted with perchloric acid, and assayed for radioactivity. Average of three experiments.

TABLE III. Effect of Actinomycin D on the Incorporation of ^{3}H-Uridine into Visna Virus–Infected PK(15) Cells.

Time after infection (hr)	Infective virus[a] ($TCID_{50}$/ml)	^{3}H-uridine (cpm coverslip)[b] Infected	Control	Percentage of control
4	6.3×10^{3}	1232	1061	116
24	3.6×10^{2}	620	586	106
48	3.6×10^{3}	384	580	66
72	6.3×10^{4}	438	617	71
96	3.6×10^{5}	837	682	123
120	1.1×10^{5}	862	809	107
168	6.3×10^{4}	456	688	66

[a] Released virus present in medium prior to addition of actinomycin D.

[b] At the time indicated monolayers were incubated with 4.0 μg actinomycin D per ml for 2 hr at 37° followed by the addition of ^{3}H-uridine at a concentration of 45 μCi/ml for 1 hr. Coverslips were then fixed, extracted with perchloric acid, and assayed for radioactivity.

stated, visna virus replicates in these cells without producing detectable cytopathic changes. As shown in Table III, little difference was found between the incorporation of the nucleoside into infected and control cells. Thus, after exposure to actinomycin D, total RNA synthesis in virus-infected cells did not appear significantly greater than in uninfected control cells.

Discussion. Attempts to incorporate radioactive nucleosides into visna virus indicate that radioactive uridine, but not thymidine, is introduced into infective virus particles. These results are in keeping with the conclusion drawn from histochemical studies of visna-infected cells (8, 9): they demonstrate that visna virus contains RNA.

The nature of the material which equilibrates at a density higher than that of infective virus and contains the largest peak of ^{3}H-uridine is not clear. The presence of a similar radioactive peak in harvests from uninfected cultures suggests that the material is a subcellular fraction. Gradient centrifugation of concentrates from infected cultures showed more radioactivity in these heavier fractions than did concentrates from uninfected cultures. This finding suggests either that (1) greater quantities of this subcellular matter are released in the destructive process caused by the virus or (2) the heavier fractions contain uncoated or fragmented viral nucleic acid. Electron microscopic and immunologic studies will be required to further characterize this material.

Actinomycin D has been shown to arrest visna virus multiplication when added to SCP cell monolayers for 2.5-hr periods from the beginning of infection until as late as 24 hr after infection (15). Similar inhibitory effects of actinomycin D have been observed on the replication of oncogenic RNA viruses (16–22) which resemble visna virus in morphology and certain biological properties (23).

The present studies on the effect of actinomycin D on RNA synthesis in visna virus–infected cells were conducted to see whether the antibiotic would selectively inhibit cellular RNA synthesis without affecting virus-specific RNA synthesis. After exposure to actinomycin D, total RNA synthesis in virus-infected cells was not significantly greater than in uninfected cells, and differences of the magnitude observed in a study of mengovirus infection (where RNA synthesis proceeds in the presence of actinomycin D) using a similar technique were not obtained (24).

Visna virus RNA, however, may be formed so slowly or inefficiently that it constitutes only a small portion of the total cellular RNA and, therefore, was not detected by the method used. Further studies will be required to determine whether virus-specific RNA can be detected in cytoplasmic extracts of infected cells and how the synthesis of such RNA is affected by actinomycin D.

Summary. Visna virus grown in sheep choroid plexus cells was concentrated by centrifugation and purified by isopynic gradient centrifugation in cesium chloride. Peak infective virus came to equilibrium at a buoyant density of 1.18–1.20 g/ml. ^{3}H-uridine and ^{32}P-orthophosphate added during infection were recovered in density-gradient fractions containing maximal amounts of infective vi-

rus. ^{3}H-thymidine added to infected cells was not detected in fractions containing infective virus. The results indicate that visna virus contains RNA.

^{3}H-uridine incorporation into virus-infected cultures previously treated with actinomycin D was not significantly greater than the incorporation of ^{3}H-uridine into uninfected cultures previously exposed to actinomycin D. This observation was made both in sheep choroid plexus cells, in which the virus produces cell destruction, and in porcine kidney cells, in which virus replication is not associated with cytopathic changes. Actinomycin D treatment failed to uncover appreciable virus-specific RNA synthesis in infected cells.

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1. Sigurdsson, B., Pálsson, P. A., and Grímsson, H., J. Neuropathol. Exptl. Neurol. **16**, 389 (1957).

2. Sigurdsson, B. and Pálsson, P. A., Brit. J. Exptl. Pathol. **39**, 519 (1958).

3. Sigurdsson, B., Thormar, H., Pálsson, P. A., Arch. Ges. Virusforsch. **10**, 368 (1960).

4. Thormar, H., Virology **19**, 273 (1963).

5. Harter, D. H. and Choppin, P. W., Virology **31**, 279 (1967).

6. Harter, D. H., Hsu, K. M., and Rose, H. M., Proc. Soc. Exptl. Biol. Med. **129**, 295 (1968).

7. Thormar, H., Virology **14**, 463 (1963).

8. Thormar, H., Acta Pathol. Microbiol. Scand. **68**, 54 (1966).

9. Harter, D. H., Hsu, K. C., and Rose, H. M., J. Virol. **1**, 1265 (1967).

10. Bablanian, R., Eggers, H. J., and Tamm, I., Virology **26**, 100 (1965).

11. Dulbecco, R. and Vogt, M., J. Exptl. Med. **99**, 167 (1954).

12. Baltimore, D. and Franklin, R. M., Biochim. Biophys. Acta **76**, 431 (1963).

13. Goldstein, M. N., Hamm, K., and Amrod, E., Science **151**, 1555 (1966).

14. Wong, K. T., Baron, S., Levy, H. B., and Ward, T. G., Proc. Soc. Exptl. Biol. Med. **125**, 65 (1967).

15. Thormar, H., Virology **26**, 36 (1965).

16. Temin, H. M., Virology **20**, 577 (1963).

17. Bader, J. P., Virology **22**, 462 (1964).

18. Vigier, P. and Goldé, A., Virology **23**, 511 (1964).

19. Zischka, R., Langlais, A. J., Rao, P. R., and Beard, J. W., Cancer Res. **26**, 1839 (1966).

20. Bases, R. E. and King, A. P., Virology **32**, 175 (1967).

21. Duesberg, P. H. and Robinson, W. S., Virology **31**, 742 (1967).

22. Yoshikura, H., Japan J. Med. Sci. Biol. **20**, 237 (1967).

23. Thormar, H., *in* "Slow, Latent, and Temperate Virus Infections," Monograph no. 2, p. 335. Natl. Inst. Neurol. Diseases Blindness, Washington, D. C. (1965).

24. Franklin, R. M. and Baltimore, D., Cold Spring Harbor Symp. Quant. Biol. **27**, 175 (1962).

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